**Materials and Methods**

**Flow chart diagram of whole work**

- **Confirmed cases of active TB**
  - **Selected TB patients**
    - **Follow up**
      - 0 day, every 15 days for the first two months, then monthly until completion of treatment
    - **Diagnosis of anti-TB-DIH by the abnormal pattern of LFTs**
      - **Patients with DIH**
        - **Anthropometric analysis**
          - Age, Sex, BMI, Socio-economic status, Marital status and Disease classification
        - **Statistical analysis**
          - Prospective cohort study
            - Find out risk factors for anti-TB-DIH by univariate and multivariate logistic regression analysis
      - **Patients without DIH**
        - **Biochemical analysis**
          - Lipid peroxidation
          - Glutathione
          - Superoxide dismutase
        - **Statistical analysis**
          - Prospective cohort study
            - Find out association between oxidative stress and anti-TB-DIH by unpaired t-test
    - **Selection of patients via inclusion/exclusion criteria**
    - **Clinically**
      - **Liver function tests (LFTs) & Oxidative stress test**
      - **Prospective cohort study**
        - Find out association between oxidative stress and anti-TB-DIH by univariate and multivariate logistic regression analysis
  - **DNA extraction**
    - **Analysis of GSTM1 gene polymorphism by PCR**
    - **Analysis of GSTT1 gene polymorphism by PCR**
    - **Analysis of CYP2E1 C1/C1, C1/C2 and C2/C2 gene polymorphisms by PCR-RFLP**

**Statistical analysis**

**Case-control study**

- Find out association between Genotypes and anti-TB-DIH by OR and 95% CI
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1. Patients
This prospective cohort study was conducted in TB patients with age ranging 15-65 years, taking anti-TB drugs under DOTS, visiting Department of Pulmonary Medicine, King George’s Medical University, Lucknow, India.

1.1. Diagnosis of TB

1.1.1. Diagnosis of Pulmonary TB
The diagnosis of Pulmonary TB was made on WHO criteria including

- Chest X-ray
- Presence of acid-fast bacilli on sputum smear
- Positive sputum culture for *Mycobacterium tuberculosis*

1.1.1.1. Chest X-ray
Chest X-ray was performed to check for lung abnormalities in patients, who had symptoms of TB disease such as persistent cough, fatigue, fever or night sweats. It is the most common diagnostic test that leads to the suspicion of infection with *M. tuberculosis*. In primary TB, a X-ray shows an abnormality in the mid and lower lung fields. However, chest X-ray cannot confirm that a person has active TB, especially if the infection is not in the lungs as in 40% of all cases of active TB. The chest X-ray also has a poor ability to detect infection in the early stages of disease.

1.1.1.2. Sputum Smear Microscopy (SSM) test
SSM test is the examination of sputum (a matter that is thrown from the lungs), for the detection of *M. tuberculosis*. This test is based on the principle of Ziehl Neelsen diagnostic technique of direct smear microscopy of sputum. The unique properties of bacterial cell wall of *M. tuberculosis* allows it to retain the primary stain even after exposure to strong acid solutions, they are called acid-fast bacteria (AFB). In the Ziehl Neelsen staining procedure, using carbol fuschsin and methylene blue, the acid-fast organisms appear red and give
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positive result (Figure 1). Positive AFB smear indicates a probable mycobacterial infection.

A negative AFB smear means that no infection is present.

Procedure (Selvakumar et al., 2002):

- A new unscratched slide was taken and labeled with the laboratory serial number.
- Sputum was spread on the slide using a broomstick.
- Allowed the slide to air dry for 15-30 minutes.
- Fixed the slide by passing it over a flame 3-5 times for 3-4 seconds each time.
- Poured filtered carbol fuchsin to cover the entire slide and was left for 5 minutes.
- Gently heated the slide with carbol fuchsin on until vapours rise.
- Gently rinsed the slide with tap water until all free carbol fuchsin stain was washed away.
- Poured 25% sulphuric acid onto the slide and let the slide stand for 2-4 minutes.
- Rinsed gently with tap water.
- Sulphuric acid was again applied for 1-3 minutes and rinsed gently with tap water.
- Poured 0.1% methylene blue onto the slide.
- Kept methylene blue on the slide for 30 seconds.
- Rinsed gently with tap water.
- Allowed the slide to dry.
- Examined the slide under the microscope using 40 x lens to select the suitable area and then examined under 100 x lens using a drop of immersion oil.
- Recorded the results in the laboratory register.
- All contaminated materials were disinfected before discarding.
Sometimes, a negative AFB smear means that the *M. tuberculosis* is not present in sufficient numbers to be seen under the microscope. A smear negative sample may still grow *M. tuberculosis* in the culture media, which allows low numbers of bacteria to multiply and be detected. Therefore, a culture test was performed to confirm the diagnosis of TB.

**Figure 1: *M. tuberculosis* bacteria using acid fast Ziehl-Neelsen stain**

1.1.1.3. Culture

Cell culturing is a method of studying bacteria by growing them on media containing nutrients. Media can be either solid media on culture plates or bottles of liquid media (culture broths). Different media are used to make it as easy as possible for the suspected microorganisms to grow. *M. tuberculosis* can be cultured (grown) from a variety of specimens and can be used to detect pulmonary as well as extra-pulmonary diseases. To isolate a single bacterial species (*M. tuberculosis*) from a mixture of different bacteria, solid media are normally used. Individual cells dividing on the surface of media, do not move away from each other, and after many replications they form visible colonies composed of tens of millions of cells all derived from a single cell. Diagnosing TB using culture take weeks because of the slow growth of TB bacilli. Culturing and identification of *M. tuberculosis* provides a definitive diagnosis of TB and can significantly increase the number of cases.
**Materials and Methods**

*Procedure:*

Modified Petroff's method was used for the culture of *M. tuberculosis* (Kiran *et al.*, 2014; Kent and Kubica, 1985).

- To 3 ml of sputum, add 3 ml of 4% NaOH (Figure 2a).
- Mixed properly using shaker and let stand for 15 min at room temperature (20–25 °C).
- Centrifuged at 3000g for 15 minutes.
- Carefully poured off the supernatant into a discard can, containing an appropriate disinfectant and resuspended the pellet.
- The pellet was neutralized by adding 20 ml of sterile distilled water.
- Again centrifuged at 3000g for 15 minutes.
- Supernatant was again removed and inoculated 0.2 ml of pellet onto two slopes of LJ medium.
- Incubated all the LJ medium slopes at 37°C.
- Observed the growth of *M. tuberculosis* for four weeks (Figure 2b-2c).
- Prepared the slides for smear microscopy and stored the sediment at 4°C or −20°C.

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**Figure 2a:** Sputum sample  
**Figure 2b:** Growth of *M. tuberculosis* on LJ Medium  
**Figure 2c:** Colonies of *M. tuberculosis*
1.1.2. Diagnosis of Extra pulmonary TB

Patients with extra pulmonary TB initially have the same symptoms as patients with pulmonary TB such as fever, night sweats and weight loss. In addition to these, they then develop complaints that are specific to the body site that has been infected with TB. When extra pulmonary TB disease is suspected on the bases of clinical symptoms, a variety of specimens are to be collected from the infected part of body through biopsy (It is a medical test that involve removal of a sample of tissue from the part of body, in order to examine for disease), and then examined under microscope through staining technique, for TB bacilli.

1.1.2.1. Pleural effusion TB

In pleural effusion TB, an infected fluid is filled in the space between the lining of the lung and the lung tissue. The diagnosis of pleural effusion TB was to be done by taking sample of infected fluid, to examine for TB bacilli. This was done by inserting a needle through the chest wall into the space between the pleura layers, and taking out some of the fluid. The fluid sample was then examined under microscope through staining technique.

1.1.2.2. Lymph node TB

This is the most common form of extra pulmonary TB. TB bacilli often infect the lymph nodes in the neck, which then swell up and the skin around them becomes inflamed. Any lymph node in the body can become infected, and often the enlarged, swollen lymph nodes cause other problems because of their size. To diagnose lymph node TB, pricked the infected lymph node with a syringe and took out a few cells and then examined under a microscope for TB bacilli.

1.1.2.3. Bones and joints TB

The most common initial symptom of bone TB is pain. TB bacilli sit in the bone or the joint, and this causes pain and swelling of the affected area. To diagnose TB of the bone or joint, X-ray, and often more sophisticated ways of X-rays like CT Scans (computer tomographic
scans) or an MRI (magnetic resonance imaging) were used. In other method, the sample of TB bacilli was to be extracted from the bone or joint, by inserting a needle in the infected bone, and then finally examined under microscope.

1.1.2.4. Central nervous system TB

In central nervous system TB, bacilli infect the brain and the spinal cord both, and cause TB meningitis. Patients with TB meningitis become very sleepy, don't react normally, cannot move their hands or feet or walk anymore, they cannot speak or focus their eyes. To diagnose it, inserted a needle into the back of the patient to access the fluid around the spinal cord (which is connected to the brain), and then examined for TB bacilli in the fluid.

1.1.2.5. TB of other places

Diagnosis of Genitourinary TB relies culturing of urine sample. Similarly, the diagnosis of Gastrointestinal TB includes CT scan and biopsy.

1.2. Selection of study subjects

Some inclusion and exclusion criteria were used to select our study subjects, from all the confirmed active TB cases including New and previous history of Relapse, Failure and Treatment after default (Patients who have already taken anti-TB drugs). These inclusion and exclusion criteria were as follows:

1.2.1. Inclusion Criteria

(i) Patients were going to start anti-TB drugs.

(ii) Not receiving any other hepatotoxic drugs parallel with anti-TB treatment.

(iii) Normal findings of liver function parameters at the beginning of the treatment.

(iv) Negative for hepatitis B/C virus infection.

1.2.2. Exclusion criteria

(i) Patients with any chronic liver disease.

(ii) Pregnancy cases.
Inadequate medical records to allow complete analysis.

1.3. **Category wise recommended treatment strategy under DOTS**

After inclusion and exclusion criteria these selected TB patients were treated with anti-TB drugs comprising of INH, RIF, PZA, ETH and STR as per the Revised National Tuberculosis Control Program under DOTS. The treatment strategy was according to their category. The treatment in Category I consists of an intensive phase of INH (H), RIF (R), PZA (Z) and ETH (E) administered under a direct supervision thrice weekly on alternate days for 2 months, followed by a continuation phase of H and R thrice weekly on alternate days for 4 months. The intensive phase of Category II consisting of STR (S), H, R, Z and E for 2 months, followed by 1 month of H, R, Z and E, was administered in the same supervised manner as Category I and was followed continuation phase consisting of 5 months of H, R and E. Category III treatment was similar to that of Category I, but was executed without an inclusion of E. Various categories of TB cases and their treatment regimens under the RNTCP are specified in Table 1.

**Table 1: Categories of TB cases and treatment strategy under DOTS**

<table>
<thead>
<tr>
<th>Category</th>
<th>Characteristics of TB cases</th>
<th>Treatment regimen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Intensive phase</td>
</tr>
<tr>
<td>Category I</td>
<td>New sputum smear-positive, seriously ill. Sputum smear-negative,</td>
<td>2(HRZE)_3</td>
</tr>
<tr>
<td></td>
<td>seriously ill, extra pulmonary.</td>
<td></td>
</tr>
<tr>
<td>Category II</td>
<td>Relapse, Failure, Treatment after default, others.</td>
<td>2(SHRZE)_3, followed by 1(HRZE)_3,</td>
</tr>
<tr>
<td>Category III</td>
<td>Sputum smear-negative, not seriously ill, extra pulmonary.</td>
<td>2(HRZ)_3</td>
</tr>
</tbody>
</table>

1.4. **Informed consent**

The study was approved by the Ethical Committee of King George’s Medical University, Lucknow, India. Patients participating in this proposed study were informed about the nature
of the study and their written consents were obtained. The questionnaire was intended to
elicit information of the patient's demographic characteristics, full details of all anti-TB drugs
with doses, concomitant use of other drugs, diseases characteristics and any clinical
manifestation of anti-TB-DIH.

1.5. Sample collection

5 ml of blood samples were taken from the patients, using disposable syringe. A total of 4 ml
of this blood was transferred into heparinised tube (acts as anticoagulant), which was used for
the estimation of oxidative stress parameters and DNA extraction. Remaining 1 ml of blood
was used to separate serum by centrifugation process for the analysis of liver function
parameters.

1.6. Criteria for the diagnosis of anti-TB-DIH

Presence of at least one of the following criteria was used to define anti-TB-DIH
(Tanaoglu, 2001):

i. A rise to more than 2 times the normal level of ALT and/or AST.

ii. A rise in total serum bilirubin over 1.5 mg/dl.

iii. Any increase in ALT and/or AST above pretreatment levels together with anorexia,
nausea, vomiting, and jaundice.

1.7. Follow up

The patients were followed up both clinically (response to therapy, any adverse effects) and
biochemically with special reference to liver function and oxidative stress parameters. Liver
function parameters included alanine aminotransferase (ALT), aspartate aminotransferase
(AST) and bilirubin. Oxidative stress parameters included malondialdehyde (MDA), GSH
and SOD. All these parameters were performed before the initiation of treatment. After the
initiation of drug treatment, follow up were performed every two weeks during the first two
month and then monthly until completion of treatment. These biochemical parameters were
repeated later, whenever symptoms suggestive of hepatotoxicity like nausea, anorexia, vomiting occurred. Proper monitoring was done in these patients and instructed to report any unusual signs and symptoms they will come across during their course. During the treatment period, only peak values of liver function parameters were recorded and at the same time level of oxidative stress parameters were also recorded.

1.7.1. Estimation of liver function parameters

Liver function parameters (ALT, AST and bilirubin) were measured in blood serum, utilizing an auto-analyzer in the pathology lab, King George’s Medical University, Lucknow, India.

1.7.1.1. ALT

ALT is mainly found in the liver, but also in smaller amounts in the kidneys, heart, muscles, and pancreas. It is also called serum glutamic pyruvic transaminase (SGPT). It is measured to see if the liver is damaged or diseased. Low levels of ALT are normally found in the blood. But when the liver is damaged or diseased, it releases ALT into the bloodstream, which makes ALT levels go up. Most increases in ALT levels are caused by liver damage. The ALT test is often done along with other tests that check for liver damage including AST, alkaline phosphatase, lactate dehydrogenase (LDH) and bilirubin. The concentration of ALT was measured in blood serum, by an auto analyzer. The normal range in the laboratory was 9-43 U/L.

1.7.1.2. AST

AST is also known as serum glutamic-oxaloacetic transaminase (SGOT). AST is a protein made by liver cells. When liver cells are damaged, AST leaks out into the bloodstream and the level of AST in the blood becomes higher than normal. AST is different from ALT because AST is found in parts of the body other than the liver including the heart, kidneys, muscles and brain. When cells in any of those parts of the body are damaged, AST can be
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elevated. The level of AST was also measured by an auto-analyzer and the normal range in the laboratory was 9-43 U/L.

1.7.1.3. Bilirubin

A bilirubin test measures the amount of bilirubin in a blood sample. Bilirubin is a brownish yellow substance found in bile. It is produced when the liver breaks down old red blood cells. Bilirubin is then removed from the body through the stool (feces) and gives stool its normal brown color. Bilirubin circulates in the bloodstream in two forms: Indirect (or unconjugated) bilirubin and direct bilirubin. Indirect form of bilirubin does not dissolve in water (it is insoluble) and travels through the bloodstream to the liver, where it is changed into a soluble form (direct or conjugated). Therefore, direct bilirubin dissolves in water (it is soluble) and is made by the liver from indirect bilirubin. Total bilirubin and direct bilirubin levels are measured directly in the blood, whereas indirect bilirubin levels are derived from the total and direct bilirubin measurements. Direct bilirubin was measured by an auto-analyzer and the normal range was 0.4-1.2 mg/dl.

1.7.2. Estimation of oxidative stress parameters

1.7.2.1. MDA

MDA concentration was determined in blood plasma by the method of Ohkawa et al. (1979). 200 μl of blood plasma was mixed with 1ml of 20% acetic acid, subsequently added 200 μl of 8% SDS (pH adjusted to 4). Following that, 1.5 ml of 0.8% thiobarbutric acid (TBA) and 1.1ml of distilled water (DW) were added. Reaction mixture was incubated in boiling water bath for one hour. After cooling, 3ml of n-butanol was added. A clear butanol fraction thus obtained was used for measuring the absorbance at 532 nm and expressed as nmol/ml.

1.7.2.2. GSH

Blood GSH was measured according to the method of Beutler et al. (1963) with slight modification. 200 μl of whole blood was mixed with 1.8 ml of cold DW and incubated for 10
min at 37 °C. 600 μl of sulphosalicylic acid was added to the reaction mixture and centrifuged at 2000 rpm for 15 min. To 200 μl of supernatant, 400 μl of phosphate buffer and 80 μl of dithiobis nitrobenzoic acid (DTNB) were added. The absorbance of the yellow color developed was measured at 412 nm and expressed as μg/ml.

1.7.2.3. SOD

SOD activity was measured according to the method of McCord and Fridovich (1969) with slight modification. 200 μl of whole blood was washed thrice with ice cold normal saline by centrifuging three times at 10,000 rpm. RBCs collected were hemolysed by adding 1.5 volume of water. The temperature was maintained at 0-4 °C by means of an ice water mixture. Hemoglobin (Hb) was then precipitated by the addition of cold ethanol (300 μl) and chloroform (180 μl). Solutions were mixed properly on each addition and then centrifuged. The resulting supernatant containing SOD was taken for the measurement of its activity. Two reactions setup were run in parallel for SOD estimation. The tube in first setup (experimental) received 1.2 ml (0.052 M) of sodium pyrophosphate, 0.3 ml (186 μM) of phenazine methosulphate (PMS), 0.3 ml (300 μM) of nitroblue tetrazolium (NBT) and 0.2 ml of enzyme source. The tubes in the second setup (reference) received the entire above reagents except the enzyme source. 0.8 ml and 1 ml DW was added respectively in both sets and finally reactions were started simultaneously by the addition of 0.2 ml (780 μM) of NADH. After an interval of 90 seconds, 1 ml of glacial acetic acid was then added to each reaction tube and absorbance was read at 560 nm against a blank on spectrophotometer. The SOD activity was expressed as Unit/g Hb.

2. Genetic analysis

To determine the possible association between anti-TB-DIH and genetic polymorphisms of drug metabolizing enzymes (GSTTI, GSTMI, CYP2E1), cases and controls were selected from our study subjects.
2.1. Selection of cases and controls

The patients, who developed anti-TB-DIH, were considered as cases. For the purpose of comparison controls were selected, from the same cohort, who showed no evidence of anti-TB-DIH.

2.2. DNA extraction

Genomic DNA was extracted from the blood samples of cases and controls using salting out procedure (Miller et al., 1988).

2.2.1. Principle

The DNA extraction method involves salting out of cellular proteins by dehydration and precipitation with saturated NaCl solution and phenol chlorophenol. Lysis buffer contains sucrose which osmolysis cells for the release of chromosomes. Sodium dodecyl sulphate (SDS) and proteinase K buffer are used for breaking disulphide bonds and protein denaturation respectively. NaCl is used for deproteinization during DNA extraction. Phenol chloroform is used for precipitation of protein. Phenol is an aromatic alcohol which selectively solubilizes lipids, proteins and cellular compounds, while leaving nucleic acids in the aqueous phase. The nucleic acid in aqueous phase are precipitated by absolute alcohol and quantified by UV-VIS spectrophotometer to check the purity of DNA.

2.2.3. Procedure

Frozen heparinised blood samples were thawed at room temperature. High molecular weight DNA was extracted by using salting out method. The following protocol was used for DNA extraction:

- 500 µl of heprinized blood was taken in a 1.5 ml micro centrifuge tube. One ml of lysis buffer was added to each tube. The contents were mixed gently by inversion and were centrifuged in a refrigerated at 10,000 rpm for 10 min.
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- The supernatant was discarded and 800 µl of lysis buffer was added to the pellet, contents were mixed gently by inversion and centrifuged at 10,000 rpm for 5 min.
- Supernatant was discarded and 300 µl of autoclaved milli Q water was added and again centrifuged at 10,000 rpm for 2 min.
- Supernatant was discarded, 150 µl of proteinase K buffer and 10 µl of SDS were added to the pellet. Frothing was done in solution with the help of a microtip.
- 200 µl of milli Q water and 100 µl of chilled NaCl solution were added and mixed gently by inversion.
- 400 µl of phenol and 100 µl of chloroform were added and after mixing, gently the solution was kept on ice for 5 min, then centrifuged at 10,000 rpm for 10 min.
- Aqueous layer was separated into a fresh 1.5 ml micro centrifuge tube, 1 ml chilled absolute alcohol was added and mixing was done by inverting the tubes. Threads of DNA appeared, it was pelleted by centrifugation at 10,000 rpm for 2 min.
- The supernatant was discarded and excess fluid was completely drained off.
- The pellet was washed twice with 70 % alcohol by centrifugation at 10,000 rpm for 5 min to remove excess salt from the pellet.
- Supernatant was discarded and the DNA pellet was dried at room temperature or 37 °C over night.
- The pellet was finally dissolved in 100-500 µl of TE buffer (on the bases of amount of DNA threads). The tubes were kept at 56 °C for at least one hour or overnight at 37 °C to dissolve the DNA. DNA samples were stored at 4 °C for immediate use or -20 °C for longer duration.

2.2.4. DNA quantification and quality analysis

After isolation of DNA, quantification and quality assessment are necessary for further analysis. This is important for many applications such as digestion of DNA by restriction
enzymes, PCR of target DNA and Real time PCR. The quality of DNA was determined by
gel electrophoresis and amount was quantified by using UV-VIS spectrophotometer.

2.2.4.1. Agarose gel electrophoresis

Principle
Electrophoresis is a technique used to separate macromolecules, especially proteins and DNA
that differ in size, charge or conformation. In this technique charged molecules are placed in
an electric field, they migrate toward either the positive or negative pole according to their
charge. DNA which have a consistent negative charge imparted by their phosphate backbone,
and migrate towards the anode.
DNA is electrophoresed within a matrix or gel. Most commonly gel is composed of agarose,
which is a polysaccharide obtained from the red algae Porphyra umbilicalis. The gel is
typically used at concentrations of 0.5 to 2% and cast in the shape of a thin slab, with wells
for loading the sample. The gel is immersed within an electrophoresis buffer that provides
ions to carry a current and to maintain the pH at a relatively constant value.

Procedure
- Prepared 1% agarose gel with Etidium bromide (EtBr) in 40 ml 1X TAE buffer.
- DNA samples (3µl) were mixed with sample buffer (with Bromophenol dye) and
  loaded onto gel.
- The gel was electrophoresed at 100 V until the dye migrated ¾ of the gel.
- DNA appeared as a single band near the well, which was visualized using a gel
documentation system.

2.2.4.2. Spectrophotometric quantification of DNA
- A260 and A280- values were noted.
- A260/A280 was used to measure nuclic acid/protein content of the DNA sample.
- A260/A280 1.8-2.0 denotes presence of nuclic acids.
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- A260/A280 < 1.8 indicates presence of proteins and/or other UV absorbers.
- A260/A280 > 2.0 indicates that samples may be contaminated with chloroform or phenol.

In either case (A260/A280 < 1.8 or > 2.0) it is advisable to precipitate the DNA.

2.3. Determination of GSTM1 and GSTT1 gene polymorphisms

To determine the presence or absence of GSTM1 and GSTT1 genes, two separate multiplex polymerase chain reactions (PCR) were performed (Wang et al., 2010; Ambreen et al., 2014).

2.3.1. Primer designing

Initially, primers were designed, using Primer 3 input software (v.0.4.0). While designing primers care as taken as follows:

- Primers were non-ambiguous (having single binding site).
- Yielded PCR products of adequate size.

After designing the appropriate primers, PCR was confirmed by electronic PCR and matched with the genomic sequence of GSTM1 and GSTT1 genes.

2.3.2. Primers, for PCR amplification of GSTM1 and GSTT1 genes

The GSTM1 and GSTT1 fragments were amplified by PCR, using primers. The details of primer sequences, annealing temperature and product size are given in Table 1.

Table 2: Primer sequences for GSTM1 and GSTT1 genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
<th>Annealing Temperature</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSTM1</td>
<td>5’ GAA CTC CCT GAA AAG CTA AAG C 3’</td>
<td>64 °C</td>
<td>230bp</td>
</tr>
<tr>
<td></td>
<td>5’ GTT GGG CTC AAA TAT ACG GTG G 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSTT1</td>
<td>5’ TTC CTT ACT GGT CCT CAC ATC TC 3’</td>
<td>66 °C</td>
<td>458bp</td>
</tr>
<tr>
<td></td>
<td>5’ TCA CCG GAT CAT GGC CAG CA 3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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2.3.3. Primers, for PCR amplification of CD36 and CYP1A1 genes

CD36 gene was used as an internal positive control for GSTM1. Similarly, CYP1A1 gene was used as an internal positive control for GSTT1 gene. The primer sequences, for PCR amplification of these genes are illustrated in Table 2.

Table 3: Primer sequences for CD36 and CYP1A1 genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
<th>Annealing Temperature</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD36</td>
<td>5’ ACT CAC CCT GAA CCC CTTC 3’</td>
<td>64 °C</td>
<td>401bp</td>
</tr>
<tr>
<td></td>
<td>5’ AGCCTCTGAGTAGTTGGG GCC 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP1A1</td>
<td>5’ ACT CAC CCT GAA CCC CT C 3’</td>
<td>66 °C</td>
<td>196bp</td>
</tr>
<tr>
<td></td>
<td>5’ AGC CTC TGA GTA GTT GGG GCC 3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.3.4. PCR

*Principle*

The PCR is an in *vitro* method for enzymatic synthesis of specific DNA sequences using two oligonucleotide primers that hybridize to apposite strands and flank the region of interest in the target DNA. A repetitive series of cycle involving template denaturation, annealing primers and extension of annealed primers by DNA Taq polymerase in the presence of dNTPs, MgCl₂ and Taq polymerase buffer. The specific region of a gene is thereby amplified up to million copies from a very small quantity of DNA.

*Procedure*

For each experiment master mix was prepared which contained all ingredients except DNA. DNA template was aliquoted in 0.2 ml PCR tubes and required volume of master mix was added to each tube (Table 3). The reaction was started with initial denaturation at 94 °C for 5 min. The details of PCR conditions are represented in Table 4.
Table 4: Components of PCR reaction mixture:

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Reagents</th>
<th>Concentration</th>
<th>Volume (µl) for one reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Autoclaved Milli Q water</td>
<td></td>
<td>3.5</td>
</tr>
<tr>
<td>2.</td>
<td>Forward Primer</td>
<td>5 pmol</td>
<td>1.0</td>
</tr>
<tr>
<td>3.</td>
<td>Reverse Primer</td>
<td>5 pmol</td>
<td>1.0</td>
</tr>
<tr>
<td>4.</td>
<td>Template (100-150 ng)</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>5.</td>
<td>Master Mix (MBI-Fermentas, USA)</td>
<td>2 X</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>Final volume</td>
<td></td>
<td>15</td>
</tr>
</tbody>
</table>

Table 5: Conditions of PCR reaction

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Stage/Condition</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Lid temperature</td>
<td>105</td>
<td>-</td>
</tr>
<tr>
<td>2.</td>
<td>Initial denaturation</td>
<td>94</td>
<td>5 minutes</td>
</tr>
<tr>
<td>3.</td>
<td>Denaturation</td>
<td>94</td>
<td>30 seconds</td>
</tr>
<tr>
<td>4.</td>
<td>Annealing</td>
<td>64-66</td>
<td>30 seconds x 35 cycles</td>
</tr>
<tr>
<td>5.</td>
<td>Extension</td>
<td>72</td>
<td>30 seconds</td>
</tr>
<tr>
<td>6.</td>
<td>Final Extension</td>
<td>72</td>
<td>10 minutes</td>
</tr>
<tr>
<td>7.</td>
<td>Hold</td>
<td>10</td>
<td>∞</td>
</tr>
</tbody>
</table>

After setting up the PCR reaction, the tubes were placed in the PCR machine as per PCR conditions. The annealing temperatures were standardized according to melting temperatures of primers sets. The number of cycles was also standardized for each PCR reaction.
2.3.6. *Agarose gel electrophoresis of PCR products*

After amplification, the PCR products were checked on agarose gel. 5 µl of amplified PCR products were mixed with 1ul 6X Xylene cyanol/bromophenol blue and electrophoresed on 2% agarose gel containing EtBr.

2.4. *Determination of CYP2E1 gene polymorphisms*

The genotypes of *CYP2E1* were grouped into three types C1/C1, C1/C2 and C2/C2. C1 was classified into wild type allele and C2 was classified into mutant allele. Primers were designed using primer 3 input software. The three genotypes of CYP2E1 were determined by PCR-RFLP (Restriction Fragment Length Polymorphism), using restriction enzyme (Wang et al., 2010; Ambreen et al., 2014). Selection of restriction enzyme was done by NEB cutter software (Figure 3).

2.4.1. *Primers, for PCR amplification of CYP2E1 gene*

Primers sequences, for the amplification of *CYP2E1* gene are illustrated in Table 5.

<table>
<thead>
<tr>
<th>Table 6: Primer sequences for <em>CYP2E1</em> gene</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gene</strong></td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td><em>CYP2E1</em></td>
</tr>
</tbody>
</table>

Materials and Methods

NCBI/Ensemble

Search for genomic sequence of gene of interest

Primer 3 input

Primer designing

NEB cutter

Selection of RE

Restriction digestion at specific site by Rsal

Digested fragment length (bp)

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Ends</th>
<th>Coordinates</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(Left End)-Rsal</td>
<td>1-352</td>
<td>352</td>
</tr>
<tr>
<td>2</td>
<td>Rsal-(Right End)</td>
<td>353-412</td>
<td>60</td>
</tr>
</tbody>
</table>

Figure 3: Primer designing and selection of restriction enzymes
2.4.2. PCR

The components and conditions of PCR for CYP2E1 gene are illustrated in Table 6 and Table 7, respectively.

Table 7: Components of PCR reaction mixture for CYP2E1 gene:

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Reagents</th>
<th>Concentration</th>
<th>Volume (µl) for one reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Autoclaved Milli Q water</td>
<td>--------</td>
<td>5</td>
</tr>
<tr>
<td>2.</td>
<td>Forward Primer</td>
<td>10 pmol</td>
<td>0.5</td>
</tr>
<tr>
<td>3.</td>
<td>Reverse Primer</td>
<td>10 pmol</td>
<td>0.5</td>
</tr>
<tr>
<td>4.</td>
<td>Template (100-150 ng)</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>5.</td>
<td>Master Mix</td>
<td>2 X</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Final volume</td>
<td></td>
<td>15</td>
</tr>
</tbody>
</table>

Table 8: Conditions of PCR reaction for CYP2E1 gene

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Stage/Condition</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Lid temperature</td>
<td>105</td>
<td>-</td>
</tr>
<tr>
<td>2.</td>
<td>Initial denaturation</td>
<td>95</td>
<td>5 minutes</td>
</tr>
<tr>
<td>3.</td>
<td>Denaturation</td>
<td>95</td>
<td>30 seconds</td>
</tr>
<tr>
<td>4.</td>
<td>Annealing</td>
<td>56.5</td>
<td>30 seconds x35 cycles</td>
</tr>
<tr>
<td>5.</td>
<td>Extension</td>
<td>72</td>
<td>30 seconds</td>
</tr>
<tr>
<td>6.</td>
<td>Final Extension</td>
<td>72</td>
<td>10 minutes</td>
</tr>
<tr>
<td>7.</td>
<td>Hold</td>
<td>10</td>
<td>∞</td>
</tr>
</tbody>
</table>
2.4.3. Selection of restriction enzyme (RE)

The PCR products were then digested with restriction enzyme. Software viz. NEB-cutter (v2.0) was used to select the restriction enzymes cutting at the site of SNPs. The conditions for restriction enzyme digestion and the fragment (allele) sizes are shown here:

<table>
<thead>
<tr>
<th>Restriction enzyme</th>
<th>Restriction enzyme digestion condition</th>
<th>Allele Sizes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rsal</td>
<td>18 h at 37 °C</td>
<td>412 bp, 352 bp, 60 bp</td>
</tr>
</tbody>
</table>

2.4.4. Restriction fragment length polymorphism (RFLP) in CYP2E1 gene

RFLP involves fragmenting of DNA by a restriction enzyme which recognizes and cuts DNA at a specific site. Restriction digestion depends on the presence of restriction enzyme specific sequences. The restriction enzyme site may or may not be present in different individuals resulting in DNA fragments of varying lengths. The DNA fragments of different length are separated by agarose gel electrophoresis. RFLP occurs when the length of a detected fragment varies between individuals. Each fragment length is considered an allele and be used in genetic analysis.

In order to detect the genotype, the following pattern was followed:

A= Complete Digestion  
B= Undigested  
C= Incomplete Digestion

Figure 4: Representation of PCR-RFLP technique
2.4.5. Process (Restriction Digestion)

10 μl of each of the PCR products were digested with the restriction enzyme *RsaI*. The digested fragments were checked by polyacrylamide gel electrophoresis.

2.4.5.1. Polyacrylamide gel electrophoresis (PAGE)

Since the molecular weight of DNA fragments digested with restriction enzymes were small, therefore, these small fragments (<200 bp) were checked on polyacrylamide gels.

A polyacrylamide gel of 1mm thickness was prepared, the gel plates were clamped and the sides were sealed with agarose. The mix was prepared in a beaker kept in ice to prevent the gel from polymerizing. APS and TEMED were freshly prepared and added slowly to the gel mix drop by drop with constant shaking. The contents were mixed properly before pouring the gel. After pouring the gel the combs were placed carefully and the gel was allowed to polymerize for 30-45 min. The clamps were removed and the gel plated with the polymerized gels were fixed to the gel holder and placed into the gel running chamber containing 1X TBE buffer. The samples were loaded and the gel was electrophorised at 100 V. After the run the gel were stained with EtBr and finally visualized by gel documentation system.

3. Statistical analysis

The data collected was entered in Microsoft Excel and checked for any inconsistency. The descriptive statistics such as mean, standard deviation and percentages were calculated. The chi-square test was used to compare categorical/dichotomous variables. The unpaired t-test was used to compare independent continuous variables. The Relative risk and its 95% confidence interval were calculated to find out the risk factors for anti-TB-DIH in univariate analysis. Further, multiple logistic regression analysis was also used to determine the adjusted risk factors for anti-TB-DIH. Odds ratio (OR) and its 95% confidence interval (CI) were also calculated to find out the association of anti-TB-DIH with *GSTM1*, *GSTT1* and *CYP2E1* genotypes. Multivariable logistic regression analysis was also used to evaluate the association.
of anti-TB-DIH with the polymorphisms of these genes, after adjusting for potential confounders. The $P$-value $<$0.05 was considered significant. All the analysis was carried out by using SPSS 15.0 version.